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Chlorophyll biosynthesis and assembly into chlorophyll–protein complexes in isolated developing chloroplasts

(5-aminolevulinic acid/chloroplast development/protein synthesis/photosynthesis)

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ABSTRACT **Isolated developing plastids from greening cucumber cotyledons or from photoperiodically grown pea seedlings incorporated ^{14}C-labeled 5-aminolevulinic acid (ALA) into chlorophyll (Chl). Incorporation was light dependent, enhanced by *S*-adenosylmethionine, and linear for 1 hr. The *in vitro* rate of Chl synthesis from ALA was comparable to the *in vivo* rate of Chl accumulation. Levulinic acid and dioxoheptanoic acid strongly inhibited Chl synthesis but not plastid protein synthesis. Neither chloramphenicol nor spectinomycin affected Chl synthesis, although protein synthesis was strongly inhibited. Components of thylakoid membranes from plastids incubated with [^{14}C]ALA were resolved by electrophoresis and then subjected to autoradiography. This work showed that (*i*) newly synthesized Chl was assembled into Chl–protein complexes and (*ii*) the inhibition of protein synthesis during the incubation did not alter the labeling pattern. Thus, there was no observable short-term coregulation between Chl synthesis (from ALA) and the synthesis of membrane proteins in isolated plastids.**

The assembly of thylakoid components during chloroplast biogenesis requires coordination between nuclear and plastid gene products and between protein and chlorophyll (Chl) synthesis (1, 2). All or most of the Chl is assembled into chlorophyll–protein (CP) complexes, which are distributed between photosystem I and photosystem II (3, 4). NaDodSO$_4$/PAGE has been used to resolve at least six major CP complexes (3, 5). The apoproteins of CP1 and CPa (components of reaction centers I and II) have been mapped on the plastid genome, while the apoprotein of the light-harvesting complex (LHC) is a nuclear gene product (6–8). The assembly and stabilization of these apoproteins requires the attachment of Chl (2, 9). Chl is synthesized within the plastid from the precusor 5-aminolevulinic acid (ALA), with the import of all the nucleus-encoded enzymes of the biosynthetic pathway (10).

The interaction between these processes has been studied primarily during the greening process *in vivo* and during the reorganization that occurs when plants are subject to different light regimes (9, 11, 12). On the basis of these studies, a scheme for the temporal assembly and stabilization of thylakoid components has been postulated (12). Typically, these processes occur over several hours and preclude the study of short-term regulatory effects and coordination between the synthesis of various thylakoid components. To examine short-term effects we utilized an *in vitro* intact plastid system derived from greening cucumber cotyledons or from photoperiodically grown pea seedlings. It has been shown that all the intermediates of Chl biosynthesis and Chl itself are synthesized in isolated plastids (13–15).

We report that these isolated plastids actively incorporate ALA into Chl at rates comparable to the rate of Chl accumulation observed *in vivo*. The newly synthesized Chl is assembled into CP complexes. The synthesis of Chl and its assembly is not diminished in the absence of protein synthesis. Conversely, protein synthesis is not inhibited by inhibitors of Chl synthesis. It appears, therefore, that, in higher plants, there is no short-term coregulation of Chl synthesis and protein synthesis at the translational level, in contrast to reports of strong coregulation in *Euglena gracilis* and in the photosynthetic bacterium *Rhodopseudomonas capsulata* (16–18).

MATERIALS AND METHODS

Plant Material. Cucumber seeds (*Cucumis sativus* var. Beit Alpha MR) were germinated on moist vermiculite at 28°C in the dark. Prior to isolation of plastids, 6-day-old seedlings were illuminated for 20 hr at 30°C (60 $\mu E \cdot m^{-2} \cdot sec^{-1}$) (1 einstein, E, = 1 mol of photons). Warm white fluorescent lights were used for all light treatments. Seeds were a gift of Moran Seeds. Pea seeds (*Pisum sativum* var. Progress No. 9) were germinated on moist vermiculite and maintained on a 12-hr light (160 $\mu E \cdot m^{-2} \cdot sec^{-1}$)/12-hr dark schedule between 21°C and 24°C. Leaflets were harvested 7 days after planting. Seeds were from Burpee.

Chloroplast Isolation. Cucumber cotyledons were homogenized with a Polytron (model PCU-2, Brinkmann) for 5–7 sec in grinding medium (330 mM sorbitol/0.2% bovine serum albumin/5 mM cysteine/1 mM $MgCl_2$/1 mM EDTA/20 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate (Tes)/10 mM Hepes, pH 7.7 at 4°C) with a tissue-to-buffer ratio of 1:4. The brei was filtered through four layers of Miracloth and centrifuged for 3 min at 2000 × *g*. The pellet was resuspended in grinding medium and layered on a discontinuous gradient of equal volumes of 30%, 60%, and 90% Percoll buffer and centrifuged for 7 min at 10,000 × *g*. Each Percoll buffer contained 3% polyethylene glycol 6000, 1% bovine serum albumin, 1% Ficoll, 330 mM sorbitol, 5 mM cysteine, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, and 10 mM Hepes, pH 7.7. Intact plastids were collected at the 60–90% Percoll gradient interface, washed once in resuspension medium (grinding medium without bovine serum albumin and cysteine), and pelleted by centrifuging for 5 min at 4000 × *g*. All steps were carried out at 4°C. Pea chloroplasts were isolated on a 30–90% continuous Percoll buffer gradient (19). Buffer composition was the same as above.

Incubation for Chl Synthesis. Incubations were for 1 hr at 30°C in a shaking water bath under white lights (60 $\mu E \cdot m^{-2} \cdot sec^{-1}$). The mixture contained plastids at a concentration of 0.3 mg of Chl per ml, 9.4 μM [^{14}C]ALA (53

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Abbreviations: Chl, chlorophyll; CP, chlorophyll–protein; LHC, light-harvesting complex; ALA, 5-aminolevulinic acid; AdoMet, *S*-adenosylmethionine; CAP, chloramphenicol; E, einstein.

mCi/mmol; 1 Ci = 37 GBq), and 1 mM *S*-adenosylmethionine (AdoMet), unless noted otherwise. Test tubes were wrapped in aluminum foil for dark incubation. After incubation, the reaction mixture was cooled rapidly to 4°C and 500 μl of acetone was added. Protein was pelleted by a 5-min spin in a table-top centrifuge and the supernatant was collected. Fifty microliters of 0.3 M NH_4OH and 300 μl of acetone were added to the pellet and the residual Chl was extracted as above. Fifty microliters of saturated NaCl solution and 2 ml of hexane were added to the pooled supernatants. After 15 min, 600 μl of the green hexane phase was mixed with 6 ml of Beckman EP Ready Solv scintillation fluid and the radioactivity was measured. Counting efficiency for ^{14}C varied between 75% and 85%.

Incubation and Assay for Protein Synthesis. One hundred microliters of the reaction mixture [containing plastids at 0.3 mg of Chl per ml and 100 μM [^{3}H]leucine (10 Ci/mmol)] was incubated for 15 min at 30°C under white lights (60 $\mu E \cdot m^{-2} \cdot sec^{-1}$). After incubation, 100 μl of 4% Triton X-100 was added and 25 μl of the solubilized plastids was spotted in duplicate on Whatman 3 MM paper discs and analyzed for leucine incorporation (20).

Chl Measurements. For measurement of rates of Chl accumulation *in vivo*, Chl was extracted from 1–2 g of cotyledons into 20 ml of 10 mM NH_4OH in 90% (vol/vol) acetone, using a Polytron. The suspension was centrifuged for 5 min at 35,000 × *g* and the supernatant was collected. Chl was reextracted from the pellet with 10 ml of ammoniacal acetone. The supernatants were pooled and Chl content was estimated (21). Chl content of intact plastids was measured by adding 5–20 μl of suspension to 1 ml of 80% acetone. Protein was pelleted and the supernatant was analyzed spectrophotometrically (21).

Thin-Layer Chromatography (TLC) of Plastid Pigments. Pigments in the hexane phase (see above) were transferred to $CHCl_3$ and separated on 0.2-mm-thick silica gel plates (Merck) (22). Subsequently, either the plates were exposed to Kodak x-ray film (XAR-5) or the bands were excised and radioactivity was measured by liquid scintillation counting.

Protein Determination. Protein content of intact plastids and thylakoids was measured by the amido black assay (23), modified as described in a 1983 Schleicher & Schuell application update.

PAGE and Autoradiography. Plastids were sedimented after incubation and washed twice with 400 μl of 100 mM Tris·HCl, pH 8.5 at 4°C, 100 mM dithiothreitol, adjusted to 2 mg of Chl per ml, and stored at −80°C. Prior to electrophoresis, thylakoid membranes were solubilized with an equal volume of 2% (wt/vol) $LiDodSO_4$, 24% (wt/vol) sucrose (final $LiDodSO_4$-to-Chl ratio, by weight, was 10:1). The Laemmli buffer system was used, substituting $LiDodSO_4$ for $NaDodSO_4$ (24). A 4% stacking gel and a 12% resolving gel were used; electrophoresis was carried out for 2 hr at 150 V (constant voltage) at 4°C in the dark. Unstained gels were dried on cellophane paper and exposed to Kodak XAR-5 x-ray film at 25°C. Marker proteins were bound to remazol brilliant blue dye so as to be visualized on unstained gels (25). Marker proteins were hemoglobin (16 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), creatine kinase (40.5 kDa), bovine serum albumin (66 kDa), and phosphorylase *b* (97.4 kDa).

Materials. Miracloth was from Calbiochem–Behring; Percoll, from Pharmacia; remazol brilliant blue, from American Hoechst (Charlotte, NC); cellophane paper from Bio-Rad; Ficoll, AdoMet, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), $LiDodSO_4$, and marker proteins, from Sigma; acrylamide, from Serva (Heidelberg); 5-amino[4-^{14}C] levulinic acid (53.2 mCi/mmol), from New England Nuclear; and L-[4,5-^{3}H]leucine (145 Ci/mmol), from Amersham (diluted to 10 Ci/mmol prior to use).

RESULTS

Isolated plastids from greening cucumber cotyledons readily synthesized Chl from ALA. In some cases, experiments were repeated with pea plastids. Table 1 shows the amount of incorporation of [^{14}C]ALA into Chl (in the hexane phase as described in *Materials and Methods*) in cucumber plastids under different incubation conditions. There was an absolute requirement for light; no incorporation occurred in the dark (Table 1, line 3). In the absence of AdoMet, required for the conversion of Mg-protoporphyrin to Mg-protoporphyrin monomethyl ester (10), incorporation was inhibited 36% (line 2). MgATP and NADPH (both at 5 mM), either separately or together (lines 4, 5, and 6) inhibited Chl synthesis; the cause of this inhibition was not investigated. Acetate (at 10 mM), a precursor of the phytol chain of Chl, did not stimulate [^{14}C]ALA incorporation (data not shown). Diuron, an inhibitor of photosystem II, inhibited Chl synthesis strongly (Table 1, line 7). This inhibition was partially reversed by the addition of ATP and NADPH (line 8), suggesting that the ATP and reducing power generated during photosynthesis are required for Chl synthesis.

To confirm that the radioactivity measured in the hexane phase was primarily due to the incorporation of [^{14}C]ALA into Chl, the pigments in this phase were separated by TLC and subjected to autoradiography (Fig. 1). Seven pigmented bands were resolved, including Chl-a and -b and four carotenoids (Fig. 1, track 1). The major radioactivity was associated with Chl-a (over 90%) and pheophytin a (a breakdown product of Chl-a) with very little radioactivity in Chl-b (track 2). No radioactivity was found with the carotenoids or at the solvent front. Similar results were obtained when the experiment was repeated with pea plastids (data not shown).

A time course study of Chl synthesis from ALA in cucumber plastids showed that incorporation of [^{14}C]ALA had a lag phase and a maximum rate was achieved around 20 min (Fig. 2*A*). Incorporation of [^{14}C]ALA between 60 and 90 min was very low. Addition of [^{14}C]ALA after 60 min did not stimulate incorporation (data not shown), indicating that the decreased rate after 60 min was not due to a lack of radioactive substrate. The average rate for the first 30 min was 6.6 pmol of [^{14}C]ALA incorporated per mg of protein per min (or 295 pmol of [^{14}C]ALA per mg of Chl per min). Under

Table 1. [^{14}C]ALA incorporation into Chl under various incubation conditions

Treatment	[^{14}C]ALA incorporated	
	pmol/mg protein	% of control
1. Control	293.7	100
2. −AdoMet	188.1	64
3. −AdoMet, dark	6.7	2
4. 5 mM MgATP	61.2	21
5. 5 mM NADPH	139.1	47
6. 5 mM MgATP, 5 mM NADPH	131.9	45
7. 1 μM diuron	23.8	8
8. 1 μM diuron, 5 mM MgATP, 5 mM NADPH	80.1	27

Cucumber plastids were incubated in the presence of additives. Incorporation of [^{14}C]ALA into Chl was measured by extracting Chl into hexane and measuring radioactivity. Results are the mean of duplicate samples. Control mixture contained 0.30 mg of Chl per ml, 1 mM AdoMet, and 9.4 μM [^{14}C]ALA.

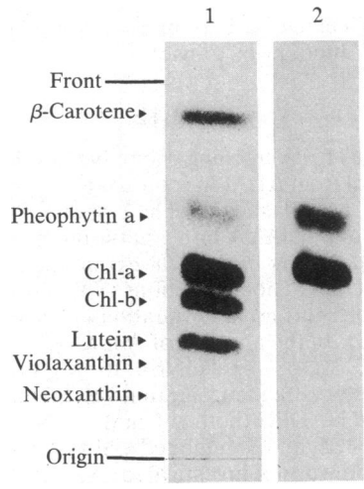

FIG. 1. Incorporation of [^{14}C]ALA into Chl by isolated cucumber plastids. Plastids were incubated with [^{14}C]ALA under standard conditions; [^{14}C]Chl was transferred from hexane into $CHCl_3$ prior to TLC. Five microliters of the $CHCl_3$ extract (8000 cpm) was spotted and developed for 7 min. Seven pigmented bands were separated as shown in track 1: β-carotene, yellow; pheophytin a, blue-gray; Chl-a, blue-green; Chl-b, yellow-green; lutein, yellow; violaxanthin, yellow; and neoxanthin, yellow. Violaxanthin and neoxanthin, being faint bands, are not visible in this photograph. Track 2, autoradiograph of track 1. Arrowheads point to the radioactive bands in track 2.

similar conditions, protein synthesis started at a linear rate, declined rapidly after 15 min, and ceased after 30 min (Fig. 2*B*). When the kinetics of incorporation in Fig. 2 *A* and *B* are compared, it appears that between 30 and 60 min Chl synthesis continued in the absence of concomitant synthesis of plastid protein.

A time course of Chl accumulation *in vivo* is shown in Fig. 3. The rate of accumulation was approximately linear between 6 and 32 hr at 0.51 μg of Chl per g (fresh wt) per min or 556.4 pmol per g (fresh wt) per min (assuming 1 mol of Chl = 898.6 g when the ratio of Chl-a to Chl-b is 2.7). The average *in vitro* rate of Chl synthesis from ALA between 10 and 30 min was 295 pmol of [^{14}C]ALA incorporated per mg of Chl per min (Fig. 2*A*), which translates to 36.8 pmol of Chl synthesized per mg of Chl per min, assuming eight molecules of ALA are required for the synthesis of one Chl molecule (10). It was determined that 4% of the total Chl in cotyledons was recovered in the intact plastids (data not shown). This recovery factor was used to express the rate of incorporation *in vitro* on a fresh weight basis. This enabled us to compare directly the rate of synthesis *in vitro* with the rate of Chl accumulation *in vivo*. The converted *in vitro* rate was 920 pmol of Chl per g (fresh wt) per min, whereas the *in vivo* rate, between 18 and 20 hr, was 556.4 pmol of Chl per g (fresh wt) per min (see Fig. 3). The average rate of Chl synthesis observed *in vitro* appears adequate to sustain the *in vivo* rate of Chl accumulation.

The effect of several inhibitors of protein and Chl synthesis was tested in cucumber plastids (Table 2). Both chloramphenicol (CAP) and spectinomycin (inhibitors of protein synthesis on 70S ribosomes) strongly inhibited protein synthesis with no significant effect on Chl synthesis (lines 2 and 3). Similar results were obtained with pea plastids (unpublished data). Inhibitors of protein synthesis on 80S ribosomes, cycloheximide and anisomycin, had virtually no effect on protein synthesis (lines 4 and 5), indicating that cytoplasmic contamination was insignificant. Both levulinic acid and dioxoheptanoic acid (competitive inhibitors of the enzyme ALA dehydratase) strongly inhibited Chl biosynthesis but had little effect on protein synthesis (lines 6 and 7).

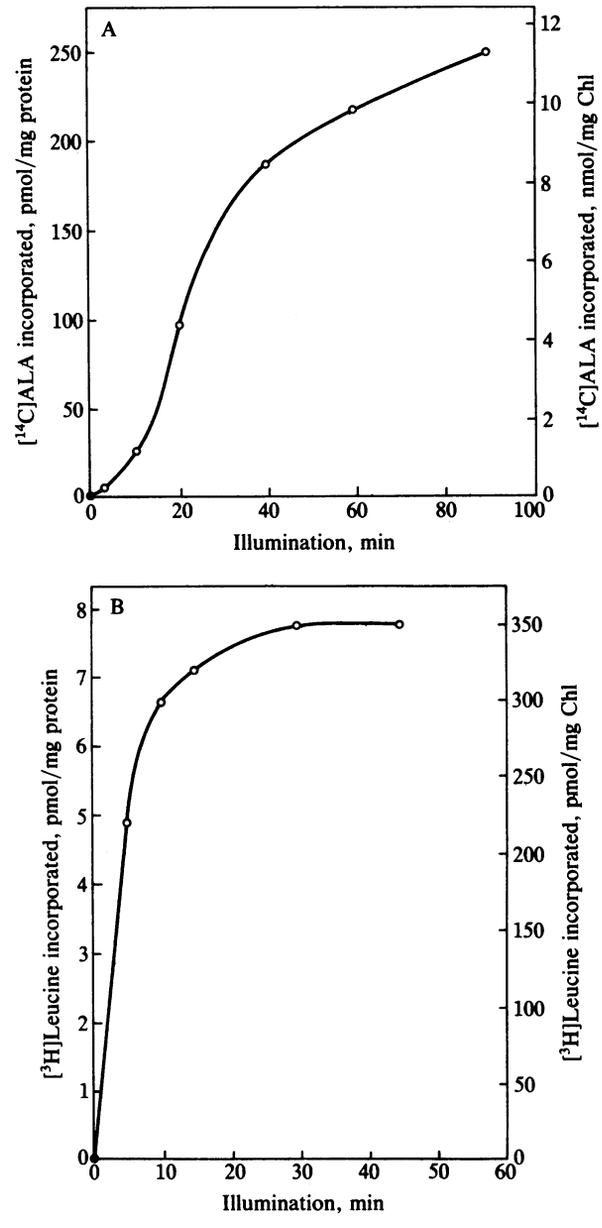

FIG. 2. Time course of Chl and protein synthesis in isolated cucumber plastids. Reaction mixtures (100 μl) containing 0.3 mg of Chl per ml, 1 mM AdoMet, and 9.4 μM [^{14}C]ALA (*A*) or 100 μM [^{3}H]leucine (*B*) were incubated for various times. [^{14}C]ALA incorporated into Chl (*A*) or [^{3}H]leucine incorporated into protein (*B*) was determined.

Neither Chl nor protein synthesis was stimulated by the addition of the 20 common amino acids (each at 200 μM) in the presence of 200 μM Mg^{2+} (data not shown).

To investigate whether the newly synthesized Chl was being assembled into CP complexes, [^{14}C]ALA-labeled membranes were solubilized and resolved by PAGE followed by autoradiography. Milder detergent treatments, reported to prevent the loss of Chl from CP complexes, were tried, but complete membrane solubilization was not achieved (4, 26). In our hands, the best results were obtained with a 10:1 (wt/wt) ratio of $LiDodSO_4$ to Chl and a final $LiDodSO_4$ concentration of 1%. Under these conditions, five pigmented bands were resolved, in addition to the free Chl at the front (Fig. 4, tracks 1 and 4). The bands were identified with respect to marker proteins and comparison with published data (3, 5). These are, in order of decreasing molecular

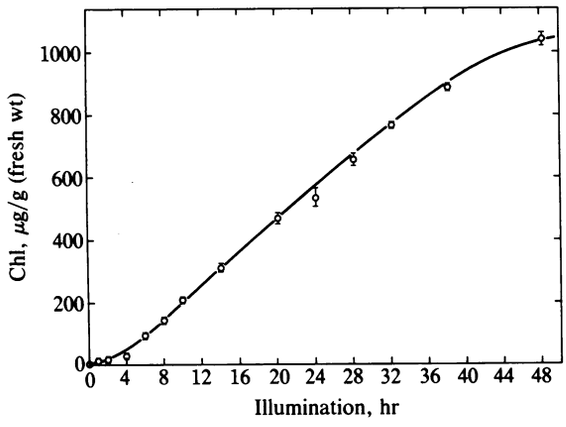

FIG. 3. Total Chl (a + b) accumulation in cucumber cotyledons during the first 48 hr of illumination. Etiolated cucumber seedlings were illuminated with white light (60 $\mu E \cdot m^{-2} \cdot sec^{-1}$) and total Chl was measured at different time points. Each point represents the mean and range of two measurements.

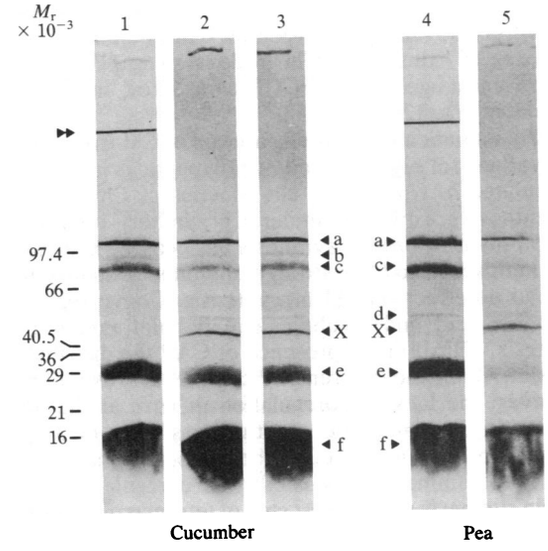

FIG. 4. LiDodSO$_4$/PAGE of [^{14}C]ALA-labeled thylakoids from cucumber and pea plastids. Track 1, unstained CP complexes from cucumber; track 2, autoradiograph of track 1; track 3, autoradiograph of thylakoids labeled with [^{14}C]ALA in the presence of 250 μM CAP. Tracks 1 and 3 were loaded with thylakoids equivalent to 10 μg of Chl (1.3×10^4 cpm). Track 4, unstained CP complexes from pea; track 5, autoradiograph of track 4 (1.2×10^4 cpm). CP complexes: a, CP1; b and c, LHC III oligomers; d, CPa; e, LHC III monomer. Band f, free Chl. Band X (seen in tracks 2, 3, and 5) does not correspond to a CP complex. Apparent molecular weights are shown at extreme left. The double arrow indicates the top of the resolving gel.

weight: CP1, oligomers of LHC III, CPa, LHC III monomer, and free Chl. The LHC oligomer was detected only in cucumber thylakoids (tracks 1 and 3), while CPa was visible only in pea thylakoids (track 4). Radioactivity was associated with all CP complexes and with free Chl (Fig. 4, tracks 2, 3, and 5). Under certain conditions, the LHC monomer (LHC III) could be separated into two radioactive bands (not shown here). Since the relative loss of [^{14}C]Chl from the CP complexes could not be estimated, the radioactivity associated with each complex was not assessed.

Track 3 of Fig. 4 shows an autoradiograph of membranes from cucumber plastids incubated with [^{14}C]ALA in the presence of CAP. The labeling pattern is identical to that in the control (track 2), which shows that Chl is assembled into CP complexes even when plastid protein synthesis is strongly inhibited. Similar results were obtained with pea plastids. The strongly labeled band X, with an apparent molecular weight of 42,000, which appears in tracks 2, 3, and 5, did not correspond to a pigmented CP band. The label was not removed by heat or ammoniacal acetone. The identity of this band has not yet been determined.

DISCUSSION

Isolated developing plastids were used to investigate the synthesis of Chl and its assembly into CP complexes. Light and AdoMet were required for the active incorporation of ALA into Chl. AdoMet donates a methyl group to Mg-protoporphyrin during its conversion to Mg-protoporphyrin monomethyl ester (10). *In vivo*, AdoMet is imported into the plastid; hence its stimulatory effect in the *in vitro* system is expected. [^{14}C]Glutamate can also be used as a precursor *in vitro*, although the rate of conversion to [^{14}C]Chl is lower than when [^{14}C]ALA is used (data not shown).

Table 2. Effect of inhibitors on protein and Chl synthesis

Treatment	[^{14}C]ALA incorporated, pmol/mg protein	[^{3}H]Leucine incorporated, pmol/mg protein
1. Control	155.2 (100)	6.1 (100)
2. 250 μM CAP	140.8 (90)	0.7 (12)
3. 500 μM spectinomycin	150.3 (97)	0.4 (7)
4. 250 μM cycloheximide	133.9 (86)	4.8 (79)
5. 120 μM anisomycin	142.1 (91)	5.7 (93)
6. 2 mM levulinic acid	18.8 (12)	4.9 (80)
7. 2 mM dioxoheptanoic acid	2.0 (1)	5.4 (89)

Cucumber plastids were incubated with [^{14}C]ALA or [^{3}H]leucine in the presence of inhibitors. CAP, chloramphenicol. Incorporation of [^{14}C]ALA into Chl and [^{3}H]leucine into protein was measured in duplicate samples. Numerals in parentheses indicate incorporation as percentage of control. Control mixture contained 0.3 mg of Chl per ml, 1 mM AdoMet, and 9.4 μM [^{14}C]ALA or 100 μM [^{3}H]leucine.

The inhibitory effect of diuron, which was partially reversed by exogenous ATP and NADPH, indicates that light provides the ATP and reducing power required for Chl biosynthesis. The synthesis of Mg-protoporphyrin and the phytylation of chlorophyllide require ATP, while NADPH is needed for at least three steps between ALA and Chl-a (10). An exogenous supply of ATP and NADPH can replace the light requirement for the synthesis of a number of Chl intermediates in isolated cucumber plastids (15).

Chl-a continues to be synthesized *in vitro* from [^{14}C]ALA for 1 hr, during which there is minimal conversion of [^{14}C]Chl-a to Chl-b. The rates of Chl synthesis *in vitro* and of Chl accumulation *in vivo* are similar. We conclude that the enzymes required for Chl synthesis are present in the plastid and remain active for at least 1 hr. However, the ability of isolated plastids to synthesize Chl is a function of the age of the plants. Plastids from 10-day-old light-grown pea or cucumber seedlings exhibited little or no Chl biosynthesis (unpublished data).

The newly synthesized Chl could either be attached to apoprotein not yet complexed with Chl or be exchanged for Chl already assembled into CP complexes (12). In the *in vitro* plastid system (Fig. 4) we can exclude the requirement for concomitant synthesis of Chl and apoprotein in the case of CP1 and CPa, which are labeled from [^{14}C]ALA in the presence of CAP, and in the case of LHC, whose apoprotein is a nuclear gene product. If we assume that these apoproteins already contained bound Chl, then our results indicate that the newly synthesized Chl is assembled into CP complexes by an exchange process. Tanaka and Tsuji (27)

reported the transfer of Chl from LHC to CP1 in cucumber cotyledons moved to the dark after 6 hr of illumination. The existence of "unstable" Chl in developing plastids, which can be exchanged between CP complexes, has also been postulated (11, 12).

Our data indicate that the synthesis of Chl from ALA and the synthesis of pigment-binding polypeptides is not strongly coregulated *in vitro*. First, the kinetics of Chl and protein biosynthesis are quite dissimilar, Chl synthesis continuing for 30–45 min after protein synthesis has ceased. Second, both spectinomycin and CAP strongly inhibited protein synthesis but had no effect on Chl biosynthesis. Conversely, dioxoheptanoic acid and levulinic acid did not inhibit protein synthesis. Third, in the presence of CAP the distribution of radioactivity in the different CP complexes was not altered. However, the lack of coregulation *in vitro* at the level of translation does not rule out the possibility of long-term coregulation, for instance, at the transcriptional level. We have not examined the possibility of coregulation between protein synthesis and the first step of tetrapyrrole synthesis—i.e., synthesis of ALA from glutamate. In this context, it is worth noting that a plastid tRNA is required for the activation of glutamate prior to its conversion to ALA (28).

This apparent lack of coregulation between Chl and protein synthesis in higher plant plastids contrasts with the results reported for *E. gracilis* and *R. capsulata* (16–18). In *R. capsulata*, levulinic acid strongly inhibited bacteriochlorophyll (BChl) synthesis as well as synthesis and assembly of the LHC (17, 18). The genes for the BChl biosynthetic pathway and for the apoproteins of the CP complexes are clustered together (29, 30), so, it is possible to envisage strong coregulation (18). Preliminary reports from isolated *E. gracilis* plastids indicate that CAP strongly inhibits both protein and Chl synthesis (16). *E. gracilis* resembles higher plants in that the assembly of CP complexes requires the interaction of the nuclear and plastid genomes (16). The difference in the regulatory mechanisms between *E. gracilis* and higher plants could confirm existing evidence of evolutionary divergence (31, 32).

It is possible that the strongly labeled band X (Fig. 4) is a cytochrome. This hypothesis is attractive because Chl and heme share a common biosynthetic pathway and also because the apoproteins of the plastid cytochromes are encoded by plastid DNA (33, 34).

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